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Induction of an Immune Response by Oral Administration of Recombinant Botulinum Toxin

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A gene encoding the full-size botulinum neurotoxin serotype C was reconstructed in vector pQE-30 and expressed at high levels in *Escherichia coli*. Three amino acid mutations (H^{229}→G, E^{230}→T, and H^{233}→N) were generated in the zinc-binding motif, resulting in complete detoxification of the modified recombinant holotoxin. The PCR-amplified wild-type light chain of botulinum neurotoxin serotype C was also expressed in *E. coli* and used as a control in all experiments. Modified recombinant holotoxin and light chain contained a histidine affinity tag at the amino terminus, which was used for detection and purification. Recombinant proteins were purified on nickel affinity resin and analyzed by Western blotting with the anti-histidine tag and anti-neurotoxin C antibodies. The results indicated that the 150-kDa molecule of modified recombinant holotoxin and the 50-kDa recombinant light chain were synthesized without degradation; however, *E. coli* did not provide for efficient nicking of modified recombinant toxin. Modified recombinant holotoxin was not toxic to mice, had no effect on nerve-evoked muscle twitch in vitro, and was not able to cleave syntaxin in crude synaptosome preparations. The recombinant light chain was also nontoxic in vivo, had no effect on evoked muscle twitch, but was able to cleave syntaxin. Modified recombinant neurotoxin and light chain were administered to animals either orally or subcutaneously. Both oral administration and subcutaneous administration of modified recombinant neurotoxin evoked high levels of serum antibodies and protective immunity. Oral administration of recombinant light chain evoked no systemic response, whereas subcutaneous administration evoked antibody production and immunity.

Botulinum toxin (BoNT), which is produced by the organisms *Clostridium botulinum*, *Clostridium butyricum*, and *Clostridium baratii*, is a remarkably potent substance that is responsible for the disease botulism (9). Although there are several different routes by which the toxin can enter the body to cause the disease, most cases are due to ingestion of toxin or to ingestion of bacteria that produce the toxin. In either case, BoNT escapes the gut to reach the general circulation (lymph and blood). It is then distributed to cholinergic nerve endings in the periphery, which are the target sites of toxin action. The toxin enters these nerves, where it acts as a zinc-dependent endoprotease to cleave polypeptides that are essential for exocytosis (6). Cleavage of these polypeptides leads to blockade of transmitter release and paralysis.

In addition to its ability to produce neuromuscular blockade, BoNT has the ability to evoke a protective immune response (5). Thus, toxin that is inactivated with formalin and administered parenterally stimulates systemic antibody production. Similarly, polypeptide fragments from the toxin that are administered parenterally can evoke protective antibodies. The fact that BoNT or fragments derived from the toxin are antigenic is not surprising. The toxin is a large molecule (molecular weight, ~150,000), and therefore one might expect that it will have many linear and/or conformational epitopes. In addition, BoNT is similar in structure and biological activity to tetanus toxin (6), and the latter is recognized as an especially immunogenic compound (5).

When one examines the clinical literature on the disease botulism and the immunology literature on the antigenic properties of the toxin, an interesting concept emerges. The clinical literature clearly demonstrates that BoNT can escape from the gut to reach the general circulation. This is an essential prelude to the ability of the toxin to reach its target organs in the periphery. The immunology literature demonstrates that toxin presented to the general circulation can evoke a protective immune response (5). Taken together, these observations give rise to the hypothesis that a derivative of BoNT that retains the ability to translocate from the gut to the general circulation but does not retain the ability to cause neuromuscular blockade could act as an oral vaccine against botulism.

In the present study, a gene encoding botulinum toxin type C (rBoNT/C) has been constructed. Site-directed mutagenesis was used to eliminate the zinc-binding histidine motif that is essential for endoprotease activity. The modified rBoNT/C holotoxin did not possess the ability to poison animals, it failed to block transmission in isolated phrenic nerve-hemidiaphragm preparations, and it did not cleave the substrate in broken-cell assays. However, when administered orally, the modified rBoNT/C holotoxin evoked systemic antibody production and immunity against native toxin.

MATERIALS AND METHODS

Reagents, clones, antibodies, and toxin. Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs (Beverly, Mass.). The expression vector pQE-30 and nickel-nitrilotriacetic acid (Ni-NTA) agarose were purchased from Qiagen (Chatsworth, Calif.). Monoclonal antibodies (MAb) specific for the 6×His affinity tag were purchased from Qiagen. Anti-syntaxin MAbs (S-0664; anti-HPC-1) were purchased from Sigma (St. Louis, Mo.), and horse anti-BoNT/C antibodies were a kind gift from the Centers for Disease Control and Prevention, Atlanta, Ga. Plasmids pCL8 and pCH3 carrying *Eco*RI fragments of BoNT/C DNA (1) were kindly provided by K. Oguma (Okayama University Medical School). Samples of native BoNT/C were kindly provided by Y. Kamata (University of Osaka Prefecture).

Construction of the plasmid expressing the BoNT/C L chain. Techniques for DNA fragment isolation, repair of overhanging ends with the Klenow fragment

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of DNA polymerase I, and ligation with T4 DNA ligase have been described in detail elsewhere (8). All cloning steps and expression were performed in *E. coli* M-15 (QIAGEN) containing the pREP4 repressor plasmid.

A DNA fragment coding for the BoNT/C L chain (rL chain) was amplified from plasmid pCL8 with the following primers: forward, 5′-CCCAATAACAATTAACAACTTTAAT-3′; and reverse, 5′-TTTCTGCAGCTATTTATTATATAATGATCTACCATC-3′, where the *Pst*I site is underlined. One cytosine was added to the 5′ end of the forward primer to provide for reconstruction of the *Bam*HI restriction site, as well as to clone light-chain DNA in frame with the pQE-30 initiation of translation methionine. In the reverse primer, a *Pst*I restriction site was introduced immediately downstream of the stop codon. The amplified product was purified, treated with T4 polymerase, cut with *Pst*I, and inserted between the Klenow-filled-in *Bam*HI and *Pst*I sites of the expression vector pQE-30 to give plasmid pQE-LC1. The structure of pQE-LC1 was confirmed by DNA sequencing.

Construction of the plasmid expressing the modified rBoNT/C holotoxin. The gene encoding the modified rBoNT/C holotoxin was assembled from three separate toxin fragments (fragments I, II, and III) generated by PCR (Fig. 1A). Initially, a DNA fragment coding for the amino-terminal portion of BoNT/C (fragments I plus II) was amplified from plasmid pCL8 in two sequential steps to generate pBot C2 (Fig. 1A). First, DNA fragment I (nucleotides [nt] 4 to 689) was amplified with the first pair of oligonucleotide primers: forward, 5′-CCCAATAACAATTAACAACTTTAAT-3′; reverse, 5′-TTTGGTACCCATTAAAATTAGTATTGGATCCAT-3′, where the *Kpn*I site is underlined. The forward primer was the same as that used for amplification of rL-chain DNA. A *Kpn*I restriction site was included in the reverse primer to generate amino acid mutations H^{229}→G and G^{230}→T at the 3′ end of fragment I (Fig. 1B). Amplified fragment I was treated with T4 polymerase, cut with *Kpn*I, and inserted between the Klenow-filled-in *Bam*HI and *Kpn*I sites of the expression vector pQE-30, resulting in plasmid pBot C1 (Fig. 1A).

Second, DNA fragment II (nt 689 to 1633) was amplified with the following oligonucleotide primers: forward, 5′-TTTGGTACCCTTAATAATGCAATGCATAATTTATATGGA-3′; reverse, 5′-GAATTCAAATAATCAACATTTTGAG-3′, where the *Kpn*I site in the forward primer and the *Eco*RI site in the reverse primer are underlined. In the forward primer, nucleotide changes were introduced to create a *Kpn*I site and generate amino acid mutations H^{229}→G, E^{230}→T, and H^{233}→N at the 5′ end of fragment II (Fig. 1B). The reverse primer was complementary to the BoNT/C sequence and contained an internal *Eco*RI site at nt 1633. Amplified fragment II was treated with T4 polymerase, cut with *Kpn*I, and inserted between the *Kpn*I and Klenow-filled-in *Sal*I sites of pBot C1. The resulting plasmid, pBot C2, contained the 5′-terminal fragment of BoNT/C (nt 4 to 1633) in frame with the ATG codon and 6×His affinity sequence of pQE-30.

DNA fragment III (nt 1633 to 3873) (Fig. 1A), coding for the carboxy-terminal domain of BoNT/C, was amplified from plasmid pCH3 with the following oligonucleotide primers: forward, 5′-TTTGAATTCTTATTATTACCTAGAATC-3′; reverse, 5′-TTTGAGCTCTTATTCACTTACAGGTACAAAAC-3′, where the *Eco*RI site in the forward primer and the *Sac*I site in the reverse primer are underlined. The forward primer was complementary to the BoNT/C sequence and contained an internal *Eco*RI site at position 1632. In the reverse primer, a *Sac*I restriction site was introduced immediately downstream of the stop codon. Amplified fragment III was digested with *Eco*RI and *Sac*I and cloned separately into *Eco*RI-*Sac*I-digested plasmid pQE-30, generating plasmid pBot C3 (Fig. 1A). Finally, the DNA encoding full-size, modified rBoNT/C holotoxin was reconstructed by introducing the *Eco*RI-*Eco*RI fragment (nt −88 to +1632) from plasmid pBot C2 into *Eco*RI-digested, calf intestine alkaline phosphatase-dephosphorylated plasmid pBot C3 to give plasmid pQE-TC1. All PCR fragments were reanalyzed by DNA sequencing.

Optimization of neurotoxin expression. PCR was used to modify a sequence of the pQE-30 vector preceding the structural gene encoding the L chain. A new forward primer, 5′-CGGTACCATGCCAATAACAATTAACAACTTT-3′, containing an additional 10 nt on the 5′ end and a new reverse primer, 5′-AGCTATAGATCTATAATAATCCAA-3′, covering the *Bgl*II restriction site (underlined) at position 892 of the BoNT/C sequence (1), were used to reamplify a DNA fragment coding the amino-terminal portion of the L chain. The amplified fragment was treated with T4 polymerase, cut with *Bgl*II, and inserted between the Klenow-filled-in *Bam*HI and *Bgl*II sites of pQE-LC1 to give plasmid pQE-LC2.

As described in Results, the synthesis of rL chain from plasmid pQE-LC2 was significantly higher than the synthesis from pQE-LC1. Therefore, the *Xho*I-*Xho*I fragment (nt −151 to +185) from plasmid pQE-LC2 was used to replace the homologous *Xho*I-*Xho*I fragment in plasmid pQE-TC1 to give plasmid pQE-TC2. The N-terminal sequences of the modified rBoNT/C holotoxin or rL chain before and after optimization of expression are shown in Fig. 2A.

Expression and purification of rL chain or modified rBoNT/C holotoxin. Cultures were grown in Lennox broth at 37°C, with shaking, to an absorbance at 600 nm A_{600} of 0.6 to 0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 1.0 mM (final concentration), and incubation was continued for an additional 5 h. Bacteria from 1 liter of induced culture were harvested by centrifugation at 4°C and resuspended in 20 ml of 50 mM sodium phosphate buffer (pH 7.4) with 300 mM NaCl. The cell suspension was lysed on ice by sonication, with two pulses of 1 min each at 75% power, with a model 60 sonic dismembrator (Fisher Scientific, Malvern, Pa.). Lysates were centrifuged at 20,000 × *g* for 30 min at 4°C. The clarified supernatants were mixed with 1 ml of packed Ni-NTA resin, incubated for 1 h at 4°C on a rotator, and finally poured into a 25-ml column. The column was washed with 30 volumes of washing buffer (50 mM sodium phosphate [pH 6.0], 300 mM NaCl, 25 mM imidazole). Bound proteins were eluted with elution buffer (50 mM sodium phosphate [pH 4.5], 300 mM NaCl). Purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoblot analysis. Proteins to be analyzed by Western blotting were separated on 10% polyacrylamide gels by the method of Laemmli (3), transferred to nitrocellulose, and processed for detection of immunoreactive proteins containing the 6×His affinity tag. Incubations with primary antibodies were performed for 1 h at 37°C with a 1:2,000 dilution of the anti-6×His affinity tag MAb or with anti-BoNT/C antibodies. Membranes were developed by enhanced chemiluminescence (Amersham Corp., Arlington Heights, Ill.) as specified by the manufacturer.

In vivo toxicity testing. The toxicities of the modified rBoNT/C holotoxin and the rL chain were tested. Modified rBoNT/C holotoxin or rL chain purified by elution from the histidine affinity resin was diluted in phosphate-buffered saline (PBS) including 1 mg of bovine serum albumin (BSA) per ml and injected intraperitoneally (i.p.) into mice. The modified rBoNT/C holotoxin or rL chain was administered in a 100-μl aliquot of PBS-BSA at a concentration of 10 μg per mouse (average weight, ~25 g). The mice were monitored for a total of 16 weeks to rule out any nonspecific toxicity. All procedures involving animals were reviewed and approved by the Thomas Jefferson University Institutional Animal Care and Use Committee.

In vitro toxicity testing. The toxicities of the rL chain and modified rBoNT/C holotoxin were compared with that of native BoNT/C. Toxicity was bioassayed on the mouse phrenic nerve-hemidiaphragm preparation (11). Tissues were excised and suspended in physiological buffer that was aerated with 95% O_2–5% CO_2 and maintained at 35°C. The physiological solution had the following composition (millimolar): NaCl, 137; KCl, 5; $CaCl_2$, 1.8; $MgSO_4$, 1.0; $NaHCO_3$, 24; NaH_2PO_4, 1.0; and D-glucose, 11; 0.01% gelatin was also added. Phrenic nerves were stimulated continuously (1.0 Hz; 0.1- to 0.3-ms duration), and muscle twitch was recorded. Toxin-induced paralysis was measured as a 50% reduction in the muscle twitch response to neurogenic stimulation.

Cleavage of substrate. Synaptosomes (1 mg/ml) were prepared as described previously (7) and incubated in the presence of modified rBoNT/C holotoxin or L chain (100 nM) for 90 min at 37°C in Tris-buffered saline (TBS) or in TBS containing 10 mM dithiothreitol. In parallel experiments, synaptosomal membranes were incubated in the presence or absence of native BoNT/C. The proteins were separated by SDS-PAGE (15% polyacrylamide), transferred to nitrocellulose, and processed for detection of immunoreactive proteins with anti-syntaxin MAb.

Immunization and sample collection. Swiss-Webster female mice (25 g; Ace Animals, Boyertown, Pa.) were immunized i.p. or per os (p.o.). For i.p. injection, each animal received 2 μg of protein in 0.1 ml of elution buffer (see above). For p.o. administration, each animal was fed 4 μg of protein in 0.2 ml of elution buffer administered through an intragastric feeding needle. The mice were immunized on day 0, and boosters were given on days 14, 28, and 42. Samples of serum from identically immunized mice were collected and pooled on days 21, 35, and 49. For collection of serum, the mice were bled through heparinized capillary tubes at the retro-orbital plexus while under isoflurane anesthesia.

Assay of serum for antibody production. Sera from immunized or control mice were assayed for antibodies by immunoblot analysis. Recombinant antigen (modified rBoNT/C holotoxin; 0.1 μg/lane) was separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat powdered milk in TBS, cut into strips, and processed for detection of immunoreactive proteins with various serum samples. Primary incubations were performed overnight (18 h) at room temperature with 1:1,000-diluted serum. A secondary horseradish peroxidase-labeled anti-mouse immunoglobulin G was used at a 1:10,000 dilution for 1 h at room temperature. After extensive washing, the membranes were developed by enhanced chemiluminescence (Amersham).

Serum neutralization assay. Native BoNT/C (10 μl, 100 ng) was incubated with 10 μl of preimmune or immune serum at 37°C for 1 h. Subsequently, the incubation mixture was diluted with 80 μl of PBS-BSA and injected i.p. The mice were monitored for 48 h to assess the residual toxicity of various mixtures.

Challenge of immunized mice with native BoNT/C. At 3 months after the third booster, immunized mice were challenged i.p. with 100 ng of native BoNT/C per animal. The survival of challenged animals was monitored for 5 days.

RESULTS

Construction of expression vectors for synthesis of rL chain and modified rBoNT/C holotoxin. A DNA fragment encoding the rL chain was amplified by PCR and ligated to vector pQE-30, generating plasmid pQE-LC1.

DNA encoding modified rBoNT/C holotoxin was reconstructed from three PCR fragments ligated into vector pQE-30, resulting in plasmid pQE-TC1 (Fig. 1A). Oligonucleotide

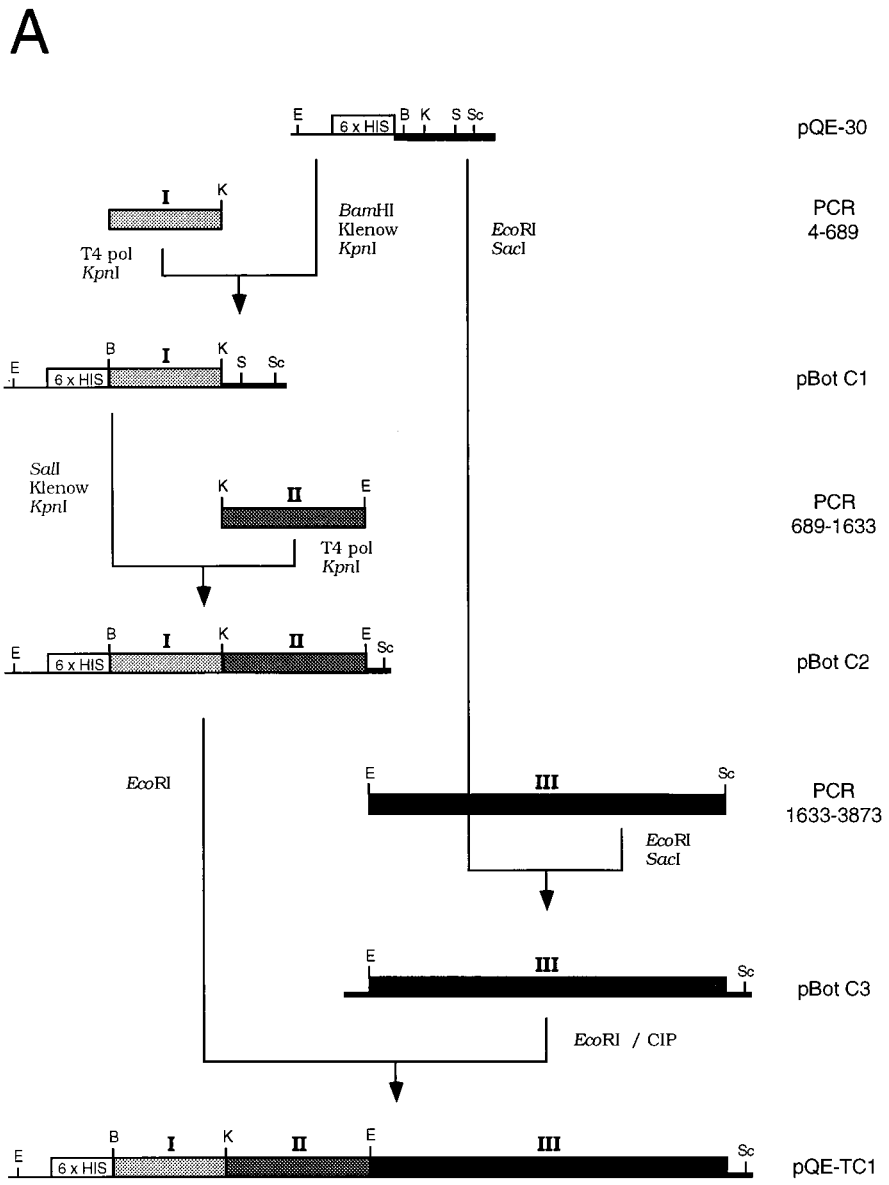

FIG. 1. Cloning of DNA encoding a modified rBoNT/C holotoxin and site-directed mutagenesis of amino acids within the zinc-binding motif. (A) DNA encoding modified rBoNT/C holotoxin was reconstituted from three PCR fragments (for details, see Materials and Methods). Fragment I (nt 4 to 689) was cloned between the *Bam*HI (B) and *Kpn*I (K) restriction sites of vector pQE-30, in frame with the 6×His affinity tag, to give pBot C1. Fragment II (nt 689 to 1633) was inserted in frame with fragment I between the engineered *Kpn*I and the blunted *Sal*I (S) restriction sites to give pBot C2. Fragment III (nt 1633 to 3873) was separately cloned between the *Eco*RI (E) and *Sac*I (Sc) restriction sites of pQE-30 to give plasmid pBot C3. Finally, an *Eco*RI fragment containing the N-terminal portion of BoNT/C (fragments I plus II) with a segment of the control area of pQE-30 was excised from plasmid pBot C2 and inserted in plasmid pBot C3 in front of fragment III encoding the C-terminal portion of modified rBoNT/C holotoxin to give plasmid pQE-TC1. (B) Three amino acids were mutated by engineering a *Kpn*I restriction site by using PCR-based mutagenesis. Numbering indicates the L-chain amino acids that have been changed (for details, see Materials and Methods).

primers were designed to engineer a *Kpn*I restriction site in the segment of DNA encoding the zinc-binding motif H^{229}-E^{230}-X-X-H^{233} (Fig. 1B). Creation of the *Kpn*I restriction site in this DNA segment accomplished two goals: (i) mutation of three amino acids (H^{229}→G, E^{230}→T, and H^{233}→N) that are essential for zinc binding, and (ii) reconstruction of a DNA encoding modified rBoNT/C holotoxin without preliminary cloning of wild-type BoNT/C DNA.

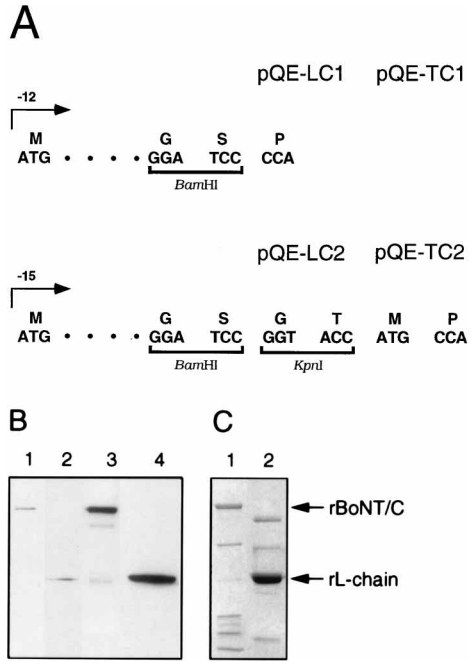

FIG. 2. Optimization of expression and purification of recombinant proteins. (A) Graphic representation of the upstream sequences of the nonoptimized (pQE-LC1 and pQE-TC1) and optimized (pQE-LC2 and pQE-TC2) expression vectors (for details, see the text). Arrows indicate the initiation of translation methionine encoded by the pQE-30 vector. The proline (P) is the first amino acid derived from native BoNT/C. (B) Western blot analysis of bacterial cell extracts expressing modified rBoNT/C holotoxin or rL chain. The synthesis of modified rBoNT/C holotoxin from pQE-TC1 and pQE-TC2 is shown in lanes 1 and 3. The synthesis of rL-chain from pQE-LC1 and pQE-LC2 is shown in lanes 2 and 4. (C) Purified proteins visualized with Coomassie blue. Unnicked modified rBoNT/C holotoxin (lane 1) and rL chain (lane 2) are shown.

The rL chain and modified rBoNT/C holotoxin, synthesized from plasmid pQE-LC1 and pQE-TC1, respectively, contained 11 additional amino acids (R G S 6×H G S) at the amino terminus (Fig. 2A).

Optimization of neurotoxin expression and purification of recombinant proteins. The ability of *E. coli* to drive the expression of the rL chain and modified rBoNT/C holotoxin from plasmids pQE-LC1 and pQE-TC1 was examined by immunoblot analysis of the cell extract. The synthesis of recombinant proteins was induced with IPTG, and aliquots of solubilized cells were subjected to SDS-PAGE. Western blot analysis with anti-6×His tag (Fig. 2B, lanes 1 and 2) or anti-BoNT/C (data not shown) antibodies revealed an extremely low level of expression for both proteins. The poor expression of the rL chain was unexpected because the L chains of serotypes A and E of BoNT were efficiently expressed in this system (data not shown). It was speculated that inhibition of neurotoxin synthesis was due to the stretch of 4 cytosines which originated from cloning of neurotoxin DNA into the *Bam*HI site of the pQE-30 vector (Fig. 2A). To check this hypothesis, new plasmids in which this cytosine sequence was deleted were constructed and designated pQE-LC2 and pQE-TC2 (Fig. 2A). Western blot analysis with anti-6×His tag antibody revealed that pQE-LC2 and pQE-TC2 were more efficient at driving the synthesis of the rL chain and modified rBoNT/C holotoxin (Fig. 2B, lanes 3 and 4). Indeed, 1 to 2 mg of modified rBoNT/C holotoxin and 5 to 10 mg of rL chain could be purified from 1 liter of Lennox broth.

rL chain and modified rBoNT/C holotoxin were synthesized in soluble form, without visible degradation, but unlike *C. botulinum*, the *E. coli* strain did not provide for efficient nicking of modified rBoNT/C holotoxin. Only trace amounts of L chain were detectable in modified rBoNT/C holotoxin by Coomassie blue staining (Fig. 2C) or Western blotting (Fig. 2B). However, modified rBoNT/C holotoxin was efficiently nicked with immobilized tolylsufonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Pierce, Rockford, Ill.) and produced heavy (H) and L chains with the correct molecular weights (data not shown). rL chain and modified rBoNT/C holotoxin synthesized from pQE-LC2 or pQE-TC2 contained 14 additional amino acids (R G S 6×H G S G T) at the amino terminus (Fig. 2A). The 6×His sequence within this 14-amino-acid segment was used for purification and subsequent detection of synthesized proteins. Recombinant proteins were purified by affinity chromatography on Ni-NTA resin with the 6×His affinity tag. Specifically bound proteins were eluted with low-pH elution buffer (pH 4.5) and analyzed by SDS-PAGE. Analysis of proteins eluted from the affinity resin revealed that both toxin fragments could be purified to a homogeneity of 80 to 90% (Fig. 2C). Recombinant proteins purified in this manner were used for all subsequent studies described in this paper.

Bioassay of recombinant proteins. Recombinant proteins were assayed for biological activity in three tests: (i) in vivo toxicity, (ii) in vitro activity on the mouse phrenic nerve-hemidiaphragm preparation, and (iii) enzymatic activity in crude synaptosome preparations.

Neither the modified rBoNT/C holotoxin with mutations in the zinc binding motif nor rL chain produced any acute toxicity in mice, even when administered at a high dose (10 μg per animal i.p.). Mice inoculated with these proteins were monitored for an additional 16 weeks, with no apparent neurotoxicity or other obvious harmful effects. Mice injected i.p. with 100 ng of native BoNT/C died within 2 to 2.5 h. This concentration of toxin represents at least 10,000 i.p. 50% lethal dose (LD_{50}) (2).

The addition of either modified rBoNT/C holotoxin or rL chain (10^{-10} M; $n = 4$) to phrenic nerve-hemidiaphragm preparations did not produce neuromuscular blockade. By contrast, addition of native BoNT/C (10^{-12} M; $n = 8$) invariably produced paralysis of transmission after 152 ± 17 min (mean ± standard error of the mean).

As expected, the rL chain retained the ability to cleave syntaxin in crude synaptosome preparations. However, the modified rBoNT/C holotoxin—whether reduced or not reduced—had no ability to cleave the peptide (data not shown).

Serum antibody response in mice immunized with modified rBoNT/C holotoxin or rL chain. Mice were inoculated p.o. with either 4 μg of modified rBoNT/C holotoxin, 4 μg of rL-chain, or TBS to assess the ability of these peptides to evoke a serum immune response. In parallel experiments, mice were given 2 μg of the same peptides subcutaneously (s.c.). All the animals were given booster doses on days 14, 28, and 42. Samples of sera were collected on days 21, 35, and 49 after immunization and analyzed by immunoblotting for immunoreactivity to unnicked modified rBoNT/C holotoxin (Fig. 3).

All mice immunized s.c. with modified rBoNT/C holotoxin had detectable antibody by day 21 (Fig. 3A, lane 3), whereas animals immunized p.o. did not (Fig. 3A, lane 1). Immunoblotting with sera collected after the second boost (day 35) still failed to show the presence of antibodies in animals immunized p.o. (Fig. 3B, lane 1). Sera collected after the third boost (day 49) demonstrated the presence of anti-toxin antibodies (Fig. 3C, lane 1).

In mice immunized s.c. with rL chain, there were no detectable serum antibodies after the first boost (Fig. 3A, lane 4) but

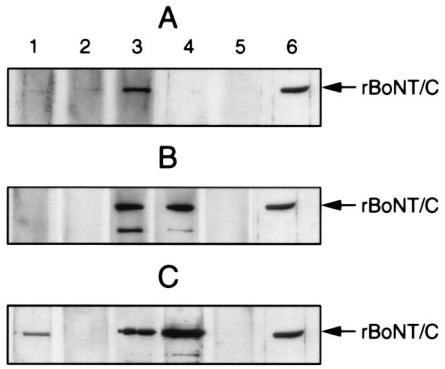

FIG. 3. Serum antibody response in mice immunized with modified rBoNT/C holotoxin or rL chain. Serum from mice inoculated p.o. or s.c. with modified rBoNT/C holotoxin or rL chain was prepared as described in the Materials and Methods. This serum was used to react with unnicked modified rBoNT/C holotoxin, which was subjected to SDS-PAGE and transferred to nitrocellulose. The L chain and H chain of unnicked holotoxin are present in the same band on gels; therefore, serum antibodies against modified rBoNT/C holotoxin or rL chain recognize the same band on a Western blot. Serum antibody responses after the first (A), second (B), and third (C) boosts are shown. Sera from mice inoculated p.o. with modified rBoNT/C holotoxin (lane 1) or rL chain (lane 2), inoculated s.c. with modified rBoNT/C holotoxin (lane 3) or rL chain (lane 4), or inoculated s.c. with buffer (lane 5) are shown. Identification of toxin with anti-His tag MAb is shown in lane 6.

antibodies were present after the second boost (Fig. 3B, lane 4). Mice inoculated p.o. with rL chain failed to produce a detectable antibody response even after a third boost (Fig. 3C, lane 2).

An additional and important observation resulted from comparing immune responses of animals that received modified rBoNT/C holotoxin p.o. with those of animals that received the toxin s.c. This observation resulted from the fact that the recombinant protein was not purified to complete homogeneity and contained some nonspecific proteins which were coadministered with modified rBoNT/C holotoxin. Interestingly, these nonspecific proteins evoked an immune response when given s.c. but not when given p.o. (data not shown).

One final observation pertained to the duration of the orally induced immune response. Animals given modified rBoNT/C holotoxin p.o. had detectable antibodies in serum for at least 3 months.

Neutralizing activity of serum from immunized mice. Experiments were done to assess the ability of various serum samples to neutralize native BoNT/C (Fig. 4A). Five different sources of serum were tested, as follows: (i) nonimmune serum, (ii) serum from animals that received rL chain either p.o. or s.c., and (iii) serum from animals that received modified rBoNT/C holotoxin either p.o. or s.c.

The toxicity of native BoNT/C incubated with nonimmune serum was equivalent to that of toxin incubated in physiological saline. The toxicity of BoNT/C incubated with serum from animals that had received rL chain either p.o. or s.c. was also similar to that of toxin incubated in physiological saline (Fig. 4A). By contrast, serum from animals that had been immunized with modified rBoNT/C holotoxin s.c. completely neutralized native BoNT/C activity (100% inhibition; Fig. 4A). Serum from animals immunized p.o. produced substantial but not complete neutralization of native BoNT/C activity (60% inhibition; Fig. 4A).

Protection of mice against challenge with native BoNT/C. Data in the preceding section indicate that mice immunized with modified rBoNT/C holotoxin should show resistance to toxin. At 3 months after the final boost, the experimental mice from which serum was obtained and tested earlier in the serum neutralization assay (see above) were challenged i.p. with native BoNT/C (100 ng per animal; Fig. 4B). As predicted, animals that received modified rBoNT/C holotoxin p.o. or s.c. were protected against challenge with native BoNT/C.

Animals that received rL chain p.o. displayed no resistance to native BoNT/C. These animals were indistinguishable from those that had received no peptide. However, animals that had received rL chain s.c. showed substantial protection against native toxin (Fig. 4B).

DISCUSSION

The development of oral vaccines that evoke systemic immunity is one of the major challenges facing investigators in-

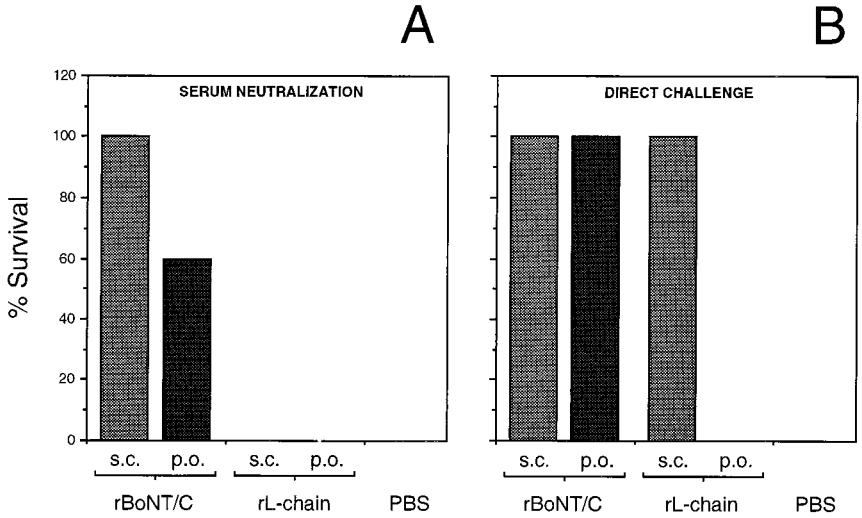

FIG. 4. Protection of mice immunized with modified rBoNT/C holotoxin or rL chain. (A) Serum neutralization assay. BoNT/C (100 ng) was incubated with serum from mice immunized with modified rBoNT/C holotoxin s.c., modified rBoNT/C holotoxin p.o., rL chain s.c., rL chain p.o., or PBS-BSA. Toxin mixtures were injected i.p. into nonimmunized mice (five animals/group), and the percent survival was determined at 48 h. (B) Direct challenge of immunized animals. BoNT/C (100 ng) was injected i.p. into mice (five animals/group) immunized with modified rBoNT/C holotoxin s.c., modified rBoNT/C holotoxin p.o., rL chain s.c., rL chain p.o., or PBS-BSA. The percent survival was determined after 48 h.

terested in the control of infectious diseases, envenomation, and various forms of poisoning (4). This task is made challenging by the fact that most potential antigens do not survive transit in the gastrointestinal system, are not efficiently transported from the lumen of the gut into the general circulation, or both. BoNT may be an exception to this general rule.

BoNT is the etiologic agent associated with the disease botulism. This disease is typically due to ingestion of food that is tainted with the toxin or to ingestion of food contaminated with organisms that can manufacture the toxin in the gut. In either case, BoNT must escape from the gut into the circulation, from which it is delivered to its target organ. This suggests that a modified version of the toxin that retains the ability to reach the general circulation but does not retain the ability to poison cells could be used as an oral vaccine.

In the present study, a modified rBoNT/C holotoxin was generated in which the zinc-binding motif of the enzymatic domain was modified in a way that abolished catalytic activity. Thus, modified rBoNT/C holotoxin containing three amino acid mutations in the zinc-binding motif was synthesized in *E. coli*; this recombinant protein lacked toxicity in vivo, failed to block neuromuscular transmission in vitro, and had no ability to cleave syntaxin in a broken-cell assay. Aside from the loss of catalytic activity and thus of neurotoxicity, the only notable difference between modified rBoNT/C holotoxin and native toxin produced by *C. botulinum* was that the former was not efficiently nicked. The inability of *E. coli* to nick modified rBoNT/C holotoxin was not expected to have a major influence on neurotoxin properties, because all serotypes of BoNT are initially produced as single-chain polypeptides that are subsequently cleaved by proteases to yield fully active two-chain molecules (9). In fact, it is the unnicked form of serotype E that is associated with human disease (10), and it is gut proteases that nick and activate the toxin.

The ability of modified rBoNT/C holotoxin to evoke an immune response was tested after p.o. administration and s.c. injection. Identical methods were used for rL chain that retained catalytic activity. As judged by immunoblot analysis, both p.o. and s.c. administration of modified rBoNT/C holotoxin evoked systemic antibody production. By contrast, the rL chain failed to evoke a response when given p.o. but did elicit a response when injected s.c. These results suggest that unlike rL chain, the modified rBoNT/C holotoxin can survive transit through the gut and/or undergo active translocation out of the gut. This conclusion is reinforced by a related finding. The modified rBoNT/C holotoxin was not purified to complete homogeneity, and thus various preparations contained a small amount of unrelated proteins. Interestingly, s.c. administration of the recombinant protein and the small contaminant evoked antibody against both proteins but p.o. administration evoked antibody against only the mutated rBoNT/C holotoxin. Again, the data suggest that only mutated rBoNT/C holotoxin possesses the characteristics that lead to translocation to the general circulation.

To this point, the data have been presented in the context that toxin escapes the gut to reach the general circulation, and this is the site at which antibody production and immunity are induced. This hypothesis is in keeping with the facts that (i) BoNT is known to escape the gut and reach the general circulation to produce its poisoning effect, and (ii) antigenic derivatives of the toxin that are administered parenterally evoke systemic antibodies and immunity. However, there is the possibility that modified rBoNT/C can evoke a local immune response in the gut and that this local response might eventually contribute to the appearance of systemic antibodies and protection. The latter idea is one that warrants further consideration.

Two types of experiments were done to estimate the protective effect of the antibodies elicited by p.o. and s.c. administration of antigens: (i) a serum neutralization test and (ii) an in vivo toxicity test. Regardless of the route of administration, serum from animals immunized with modified rBoNT/C holotoxin inactivated a large dose (~10,000 LD_{50}) of native BoNT/C. Serum from animals immunized with rL chain produced no significant reduction in the apparent potency of 10,000 LD_{50}.

For the in vivo toxicity test, immunization with modified rBoNT/C holotoxin by either the p.o. or s.c. route produced a dramatic reduction in the potency of a subsequent injection of native toxin. A similar result was obtained when the rL chain was given s.c. but not p.o. The apparent difference in outcome in the serum neutralization test and the in vivo toxicity test following s.c. administration of the rL chain may simply be a matter of titration. The small serum aliquot contained only a fraction of the antibodies present in the live animal.

In conclusion, these results provide the first demonstration of expression of an rBoNT/C holotoxin. The data further show that a modified rBoNT/C holotoxin can be constructed and expressed in a form that is devoid of toxicity. The nontoxic, modified rBoNT/C holotoxin retained the ability to translocate from the gut to the general circulation and to evoke protective antibodies. Thus, this molecule is an effective oral vaccine against BoNT/C.

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